ISSN 0126-0421
Bul. FKH-UGM Vol XV No. 1 dan 2: 38-45
Juli - Desember 1996

2513

# RADIO RECEPTOR ASSAY FOR DIAGNOSTIC SERUM AND MILK 1,25-DIHYDROXYCHOLECALCIFEROL

Hastari Wuryastuti*

## ABSTRACT

**The objectives of this study were to modify and develop technique for determination of 1,25-$(OH)_2D_3$ concentrations in sera and milk samples. The developed technique, used non-HPLC for extraction and purification of the samples and involved *radio receptor assay* which used calf thymus as 1,25-$(OH)_2D_3$ receptor for quantitation of 1,25-$(OH)_2D_3$. Results of this study were compare to that of the technique developed by Horst *et al.* (1981) which used HPLC for samples extraction and purification. The correlation between the two techniques was r=0,95. Using the modified technique the 1,25-$(OH)_2D_3$ concentrations in cow and sow sera were $57.42 \pm 5.3$ pg/ml (n=20) and $75.22 \pm 6.05$ pg/ml (n=20) respectively, whereas the 1,25-$(OH)_2D_3$ concentrations in cow and sow milk were $16.62 \pm 2.2$ pg/ml (n=20) and $21.52 \pm 3.125$ pg/ml (n=20). Based on the results could be concluded that the technique that had been developed in this study was found satisfactory for determination the 1,25-$(OH)_2D_3$ concentration either in serum or milk samples.**

* Veterinary Clinic Department, Faculty of Veterinary Medicine, GMU.

## ABSTRAK

Penelitian ini bertujuan memodifikasi dan mengembangkan teknik pengukuran konsentrasi 1,25-$(OH)_2D_3$ di dalam serum dan susu. Teknik yang dikembangkan tanpa menggunakan *HPLC* untuk ekstraksi maupun purifikasi sampel dan melibatkan *radio receptor assay* menggunakan kelenjar thymus anak sapi sebagai reseptor 1,25-$(OH)_2D_3$. Hasil dari penelitian ini dibandingkan dengan teknik yang telah dikembangkan oleh Horst *et al.* (1981) yang menggunakan HPLC untuk ekstraksi dan purifikasi sampel. Korelasi antara kedua teknik (r=0,95). Menggunakanan modifikasi teknik, konsentrasi 1,25-$(OH)_2D_3$ di dalam serum sapi dan babi masing-masing adalah $57,42 \pm 5,3$ pg/ml (n=20) dan $75,22 \pm 6,05$ pg/ml (n=20) sedangkan konsentrasi 1,25-$(OH)_2D_3$ di dalam susu sapi dan babi masing-masing sebesar $16,62 \pm 2,2$ pg/ml (n=20) dan $21,52 \pm 3,125$ pg/ml (n=20). Dari hasil dapat disimpulkan bahwa teknik yang dikembangkan dalam penelitian ini cukup memuaskan untuk penentuan konsentrasi 1,25-$(OH)_2D_3$ baik di dalam serum maupun susu.


## INTRODUCTION

1,25-dihydroxycholecalciferol (1,25$(OH)_2D_3$) is the most active metabolite of vitamin $D_3$ (DeLuca, 1981). It is considered as a steroid hormone produced by the kidney. Measurement of (1,25$(OH)_2D_3$) in serum is helpful in the diagnosis and management of various diseases associated with vitamin D and mineral metabolism disorders such as: parathyroid gland disorders; renal failure; certain bone diseases; sarcoidosis; parturient hypocalcemia etc. Because changes in circulating 1,25$(OH)_2D_3$ are physiopathological importance in many diseases, the development of an assay that is specific for and sensitive to 1,25$(OH)_2D_3$ is really required. Several different assay techniques namely high performance liquid chromatography (HPLC) (Jones, 1978); bioassay (Stern *et al.* 1978) and radio immunoassay (RIA) (Clemen *et al.* 1980; Bouillon *et al.*, 1980) have been reported previously. However, the methods frequently involve difficult extraction's, very laborius procedures and require relatively large amount of sample. The objective of this study was to find techniques which could reduced the high technical and instrumentational and total amount of samples requirement. A assay which was developed by Reinhardt *et al.* (1984) combined with nonequilibrium competitive binding assay seemed to meet these criteria and was adapted to the assay of serum and milk.

## MATERIAL AND METHODS

**Sample extraction.** Sera and milk collected from 20 cows and 20 sows were used as samples. Half to four millilitres of each sample was pipetted into a 12 x 75 mm borosilicate glass tube. Fifty mikroliters of ethanol buffer containing 1700 disintegrations per minute (DPM) [$^3$H] 1,25$(OH)_2D_3$ were added to tube and also to a scintillation vial containing 5 ml scintillition liquid for calculating recoveries. One volume of acetonitrile was added to each sample and each tube was then vortexed for 5 seconds and centrifuged for 10 minutes at 760xg. While the samples were being centrifuged, Sep-Pak $C_{18}$ columns were prepared by washing with 5 ml acetonitrile followed by two 5 ml washed distilled water.

After centrifugation, the supernatant was poured off into another 12x75 mm glass tube containing 0.5 volume of 0.4 M potassium phosphate (pH 10.5) and vortexed for 5 seconds. This extract was then applied with a pasteur pipette into the washed Sep-Pak $C_{18}$ columns. Excess salt and pigments were removed from the columns by washing twice with distilled water and the interfering polar lipids were removed by washing with methanol: distilled water (70:30). The purified vitamin D metabolite was then eluted with 5 ml acetonitrile and the elutee were evaporated to dryness using a vacuum evaporator.

After the eluates had dried, each samples was reconstituted with 5 ml of hexane:isopropanol (98:2), mixed well by vortexing and applied to a Sep-Pak Silica column. The column was prepared before use by washing with 5 ml of isopropanol followed by 2 washes of 5 ml of hexane:isopropanol (98:2). Each eluate tube was rinsed with an additional 5 ml of hexane:isopropanol (98:2), vortexed for 5 seconds, and the rinse was applied to the Sep-Pak Silica column. The25-(OH)$D_3$ and 24,25$(OH)_2D_3$ were removed from the Sep-Pak Silica column by washing with 5 ml of hexane:isopropanol ((4:6). The purified 24,25$(OH)_2D_3$ was eluated from the silica column with 5 ml of hexane:isopropanol (70:30) and dried in a vacuum evaporator.

The dried samples containing the 1,25$(OH)_2D_3$ fraction were immediately reconstituted with 200 $\mu$l ethanol buffer. From this volume, 50

$\mu$l of purified samples were used to determine the recovery sample and two 50 $\mu$l aliquates were used for competitive binding assay.

**Preparation of receptor.** Fresh bovine thymus glands were obtained from a slaughterhouse, washed ice-cold saline, cut into small cubes and homogenized (25%, w/vol) in a buffer containing 50 mM TrisHCl mM KCl, 5 mM dithiothreitol, 10 mM $Na_2MoO_4.2H_2O$ and 1.5 mMEDTA, pH 7.5. The homogenate was centrifuged for 1 h. at 300,00 x g. The supernatant was fractionated by $(NH_4)2SO_4$ to 38% saturation and centrifuged for 20 min at 20,000 x g. The supernatant was discarded, and the pellet was dissolved in buffer described above, lyophilized and the stored at-20°C until use.

**Competitive binding assay.** Fifty mikroliters of each $1,25(OH)_2D_3$ standard (25, 50, 100, 200 and 400 pg/tube) and samples were added to the 12x75 mm borosificated glass tube. Four hundred mikroliters of calf thymus receptor in phosphate potassium chloride buffer were added to each standard and sample. These mixture were then vortexed for 5 sec, and incubated in a 15-20°C water bath for 60 sec. At the end of this period, 11,500-13,500 DPM of [$^3$H] $1,25(OH)_2D_3$ in 50 $\mu$l ethanol buffer were added to each tube. The tube were mixed well and incubated again for 60 min. at 15-20°C. Following this incubation, the tubes were placed in an ice bath for 10 min to cool and then each tube received 100 $\mu$l charcoal suspension. The tubes were vortexed for 5 sec. And placed for 20 min. in an ice bath. After cooling, the tubes were vortexed again for 50 sec. And centrifuged for 15 min. at 4°C at 1800 xg. Following centrifugation, the supernatant was poured off into a scintillation vial containing 5 ml scintillation fluid. The vial of supernate and scintillation fluid was mixed well by hand and the placed in a $\beta$-scintillation counter. Data as expressed as mean counts per minute.

**Calculation.** The $1,25(OH)_2D_3$ value was calculated using a logit/log plot of the data and the concentration (picograms per ml) of serum or milk were obtained by correcting the picograms per tube data, obtained by the program, for recovery and sample volume.

**Standard assay used for comparisons.** The method of Horst *et al.* was used as the standard to which our assay was directly compared.

## RESULTS AND DISCUSSION

The importance of $1,25(OH)_2D_3$ in calcium homeostasis requires the development of improved procedures for the measurement of this metabolite. Previous assay procedures generally involve lenghty and cumbersome extraction procedures and require large volumes of samples and solvent. In the assay we modified the extraction procedures developed by Turnbull *et al.* (1981). Serum and/or milk samples (0.5-0.4 ml) were mixed with 1 vol. Of acetonitrile to remove denatured protein and insoluble lipids. The addition of 0.4 M $K_2HPO_4$ buffer to the acetonitrile extract in additional removal of lipids. Purification of the acetonitrile-$KH_2PO_4$ buffer-treated sample on a $C_{18}$ Sep-Pak column extracted and partially purified $1,25(OH)_2D_3$. Washing the C18 Sep-Pak column before elution of the vitamin D metabolite removes most of the plasma lipids and pigments and reduces the recovery of greater than 85% of the vitamin D metabolites. The results obtained were then compared with standard assay which involved HPLC for samples extraction and purification. There was a good correlation (r=0.95) between the two techniques (Fig. 1) Therefore, this step eliminated the need for the time-consuming and large solvent demands of Sephadex LH-20 bath column which are used in HPLC procedures.

After solid phase extraction. $1,25(OH)_2D_3$ was further purified on a Silica Sep-Pak. This final purification is very simple and inexpensive, because requires only Silica Sep-Pak and hexane:isopropanol solvent systems to effect elution and purification of the most active metabolite form of vitamin D. Recovery during the extraction stage of the procedures was done to validate the assay and as determined by comparing radioactivity present in lipid extracts to radioactivity added to serum or milk samples. Recoveries were 94.9% for $1,25(OH)_2D_3$ in serum (n=40) and 93.6% for $1,25(OH)_2D_3$ in milk (n=40).

Assay sensitivity was optimized by using a nonequilibrium protein binding assay adapted from Radio immuno Assays. Maximum specific binding and sensitivity were obtained by preincubation of standard and samples with bovine thymus cytosol a specific receptor for $1,25(OH)_2D_3$. The lyophilized receptor was very stable for up to 1 year and could supplies more than 5000 assays. However, after reconstitution of the receptor using distilled or deionized water, the stability would be reduced by repeatedly freezing and thawing. Separation of bound and free $1,25(OH)_2D_3$ by dextran coated charcoal was optimal at 20 min. and decreased thereafter. Assay sensitivity

was also tested by using different amount of samples from the same pools (Table 1.) The results showed linearity over a wide range of volumes.

## CONCLUSION

From the results can be concluded that radio receptor assay has several advantages: high sensitivity; small sample requirements; inexpensive, eliminate the need for HPLC and found satisfactory for determination the $1,25(OH)_2D_3$ concentrations either in serum or milk samples.

## REFERENCES


Bouillon R, P De Moor, EG Baggiolini and MR Usskokovic. 1980. A radioimmunoassay for 1,25-dihydroxycholecalciferol. Clin. Chem. 25:562.

Clemens TL, GN Hendy and SE Papapoulos. 1980. Measurement of 1,25-dihydroxycholecalciferol in man by radioimmunoassay. Clin. Endocrinol. 11:225.

DeLuca HF. 1981. The vitamin D system: A view from basis science to the clinic. Clin. Biochem. 14:213.

Horst RL, EL Littledike, JL Riley and JL Napoli. 1981. Quantitation of vitamin D and its metabolites and their plasma concentrations in five species of animals. Anal. Biochem. 116:189.

...es MJM. 1978. Analysis for 1,25-dihydroxyvitamin D in human plasma, ...r a liquid chromatographic purification procedures, with a ...mpetitive protein-binding assay. Clin. Chem. 22:450.

..., JW Orf and BW Hollis. 1984. A Microassay for ...amin D not requiring high performance liquid ... application to clinical studies. J. Clin. Endocrinol.

Stern PH, AJ Hamstra, HF DeLuca and NH Bell. 1978. A bioassay capable of measuring 1 picograms of 1,25-dihydroxyvitamin D3. J. Clin. Endocrinol. Metab. 46:891.

Turnbull H, DJH Trafford and HLJ Makin. 1981. A rapid and simple method for the measurement of plasma 25-OHD2 and 25-OHD3 using Sep-Pak cartridges and a single high performance liquid chromatography step. Clin. Chem. Acta 120:65.


(S.T.)

Table 1. The 1,25-dihydroxycholecalciferol concentration in varying volumes of samples from the same pool

| Type of sample | Sample volume (ml) | Measured value (n=20) | Concentration (pg/ml) |
|---|---|---|---|
| Cow Serum | 0.5 | 29.1 ± 2.0 | 58.2 |
| | 1.0 | 56.3 ± 2.9 | 56.3 |
| | 2.0 | 115.8 ± 6.1 | 57.9 |
| | 4.0 | 229.3 ± 10.2 | 57.3 |
| Cow Milk | 0.5 | 8.7 ± 1.0 | 17.4 |
| | 1.0 | 16.9 ± 1.9 | 16.9 |
| | 2.0 | 32.8 ± 3.1 | 16.4 |
| | 4.0 | 63.4 ± 2.8 | 15.8 |
| Sow Serum | 0.5 | 35.6 ± 3.2 | 71.2 |
| | 1.0 | 77.2 ± 1.8 | 77.2 |
| | 2.0 | 152.8 ± 3.8 | 76.4 |
| | 4.0 | 304.4 ± 15.4 | 76.1 |
| Sow Milk | 0.5 | 11.0 ± 1.9 | 22.0 |
| | 1.0 | 22.8 ± 2.6 | 22.8 |
| | 2.0 | 39.9 ± 5.4 | 19.9 |
| | 4.0 | 85.5 ± 2.6 | 21.4 |

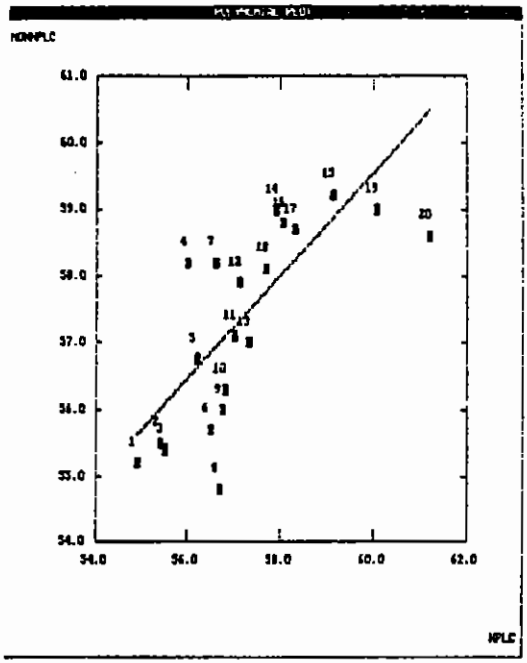


**Figure 1. Comparison of $1,25(OH)_2D_3$ concentration obtained using non-HPLC and HPLC purification before assay.**